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Extraordinarily wide genomic impact of a selective sweep associated with the evolution of sex ratio distorter suppression

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1 **Extraordinarily wide genomic impact of a selective sweep associated with the evolution of sex**

2 **ratio distorter suppression**

3

4 Running head: Genomic impact of intense selection in a butterfly

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21

Abstract

Symbionts that distort their host's sex ratio by favouring the production and survival of females are common in arthropods. Their presence produces intense Fisherian selection to return the sex ratio to parity, typified by the rapid spread of host 'suppressor' loci that restore male survival/development. In this study, we investigated the genomic impact of a selective event of this kind in the butterfly *Hypolimnas bolina*. Through linkage mapping we first identified a genomic region that was necessary for males to survive *Wolbachia*-induced killing. We then investigated the genomic impact of the rapid spread of suppression that converted the Samoan population of this butterfly from a 100:1 female-biased sex ratio in 2001, to a 1:1 sex ratio by 2006. Models of this process revealed the potential for a chromosome-wide selective sweep. To measure the impact directly, the pattern of genetic variation before and after the episode of selection was compared. Significant changes in allele frequencies were observed over a 25cM region surrounding the suppressor locus, alongside generation of linkage disequilibrium. The presence of novel allelic variants in 2006 suggests that the suppressor was introduced *via* immigration rather than through *de novo* mutation. In addition, further sampling in 2010 indicated that many of the introduced variants were lost or had reduced in frequency since 2006. We hypothesise that this loss may have resulted from a period of purifying selection - removing deleterious material that introgressed during the initial sweep. Our observations of the impact of suppression of sex ratio distorting activity reveal an extraordinarily wide genomic imprint, reflecting its status as one of the strongest selective forces in nature.

43 **Author summary**

44 The sex ratio produced by an individual can be an evolutionary battleground. In many arthropod

45 species, maternally inherited microbes selectively kill male hosts, and the host may then in turn

46 evolve strategies to restore the production or survival of males. When males are rare, the intensity

47 of selection on the host may be extreme. We recently observed one such episode, in which the

48 population sex ratio of the butterfly *Hypolimnas bolina* shifted from 100 females per male to near

49 parity through evolution of a suppressor gene. In our current study, we investigate the hypothesis

50 that the strength of selection was so strong in this case that the genomic impact would go well

51 beyond the suppressor gene itself. Following location of the suppressor within the genome of *H.*

52 *bolina*, we examined changes in genetic variation at sites on the same chromosome as the

53 suppressor. We show that an extraordinarily wide area of the genome was affected by the spread of

54 the suppressor. Our data also suggest that the selection may have been sufficiently strong to

55 introduce deleterious material to the population that was later purged by selection.

56

57   **Introduction**

58   In 1930, Fisher noted that the strength of selection on the sex ratio was frequency dependent [1]. As

59   a well-mixed outbreeding population progressively deviates from a 1:1 sex ratio, selection on

60   individuals to restore the sex ratio to parity becomes stronger. In natural populations, a principle

61   cause of population sex ratio skew is the presence of sex ratio distorting elements, in the form of

62   either sex chromosome meiotic drive [2], or cytoplasmic symbionts [3]. In some cases, these

63   elements can reach very high prevalence, distorting population sex ratios to as much as 100 females

64   per male [4], and producing intense selection for restoration of individual sex ratio to 1 female per

65   male. The most common consequence of this selection pressure is the evolution of systems of

66   suppression – host genetic variants that prevent the sex ratio distorting activity from occurring.

67   Suppressor genes are known for a wide range of cytoplasmic symbionts and meiotic drive elements

68   [2,5,6].

69

70   The evolution of suppression of *Wolbachia* induced male-killing activity in the butterfly

71   *Hypolimnas bolina* represents a compelling observation of intense natural selection in the wild.

72   Female *H. bolina* can carry a maternally inherited *Wolbachia* symbiont, wBol1, which kills male

73   hosts as embryos [7]. The species also carries an uncharacterised dominant zygotically acting

74   suppression system that allows males to survive infection [5]. Written records and analysis of

75   museum specimens indicate this symbiont was historically present, and active as a male-killer,

76   across much of the species range, from Hong Kong and Borneo through to Fiji, Samoa and parts of

77   French Polynesia [8]. Evidence from museum specimens also indicates that host suppression of

78   male-killing had a very restricted incidence in the late 19th century, with infected male hosts (the

79   hallmark of suppression) being found in the Philippines but not in other localities tested. By the late

80   20th Century, suppression of male-killing was found throughout SE Asia, but not in Polynesian

81   populations where the male-killing phenotype remained active [9]. The most extreme population

82   was that of Samoa, where 99% of female *H. bolina* were infected with male-killing *Wolbachia*,

4

83 resulting in a population sex ratio of around 100 females per male [4]. However, following over 100

84 years of stasis on Samoa, rapid spread of suppression of male-killing activity of the bacterium was

85 finally observed between 2001 and 2006, restoring both individual and population sex ratio to parity

86 [10].

87

88 When selection occurs at a locus, it is expected to leave a genomic imprint beyond the target of

89 selection, as a result of genetic hitch-hiking. During the spread of a beneficial mutation, any variant

90 that is initially associated with the selected locus through linkage will also increase in frequency

91 [11]. When selection is strong, such selective sweeps may increase the frequency of linked variants

92 across a broad genomic region [12]. Importantly, the extent of a sweep will most depend on the

93 selection pressure in the first few generations, before recombination has broken down associations

94 between the target of selection and linked variants. Where sex ratio distorters are common, the

95 selection pressure in these first generations may be very strong indeed. It is thus likely that selection

96 on the sex ratio will influence linked material over an extraordinarily broad genomic region, as

97 compared to many other selective regimes. That is, the episode of selection is likely to have a very

98 wide genomic impact.

99

100 In this paper, we first mapped a genomic region in SE Asian butterflies that was required for male

101 survival in the presence of *Wolbachia*. We then investigated the impact of the recent spread of the

102 suppressor in Samoa on the pattern of variation around this region. To this end, we initially

103 developed theory to predict the impact of suppressor spread on linked genetic variation. We then

104 directly observed changes in the frequency of genetic variants surrounding the suppressor locus by

105 comparing the pattern of genetic variation in *H. bolina* specimens collected in Samoa before (2001)

106 and after the sweep (2006 and 2010). By examining post-sweep samples at two time points we were

107 additionally able to track allele frequency changes following the initial sweep. The data revealed the

108 broadest sweep recorded to date in nature. We further suggest that the suppressor was likely derived

5

109   through immigration, and that the sweep may have introduced deleterious material subsequently

110   subject to purifying selection.

111

112   **Results and Discussion**

113

114   ***Location of a region required for male survival in the H. bolina genome***

115

116   *Hypolimnas bolina* has 31 chromosomes and a total genome size of 435 MB [13] [14]. Genetic

117   markers spanning the genome were developed using a targeted gene approach informed by

118   conservation of synteny in Lepidoptera, with the sequence of *H. bolina* orthologs obtained through

119   Roche 454 transcriptome sequencing (see Methods and Materials). These markers were then tested

120   for co-segregation with suppression in order to identify the linkage groups associated with male

121   host survival. Female butterflies from SE Asia that carried both the *Wolbachia* and the suppressor

122   allele, were crossed with males from the French Polynesian island Moorea (where suppression is

123   absent). These resulting F1 daughters (who inherited *Wolbachia* from their SE Asian mother) were

124   then backcrossed to Moorea males to create a female-informative family for identification of loci

125   linked to the suppressor. The absence of recombination in female Lepidoptera means a SE Asia

126   allele necessary for male survival that is present in the F1 mother will be present in all her sons (as

127   if they lack it, they die), but show normal 1:1 segregation in her daughters (Fig. 1). From 8 initial

128   genomic loci screened, one locus orthologous to sequence on chromosome 25 in the moth *Bombyx*

129   *mori* showed this pattern of inheritance whereby all 16 sons carried the same maternal allele of SE

130   Asia origin while 8 daughters showed Mendelian segregation at this locus (probability of observing

131   this pattern of segregation in sons $= (1/2)^{16}$: p<0.0001). We then obtained a further 11 markers in

132   this linkage group. Candidates were identified initially via synteny to *B. mori*, and then confirmed

133   as showing co-segregation with the original marker and as being associated with male survival, in

134   the female-informative family. In this way, a suite of 12 suppressor-linked markers (A-L) were

135   developed, all of which followed the presumed pattern of inheritance of the suppressor - that of

136   presence in all 16 sons and half daughters.

137

138   A linkage map for this chromosome was then constructed, with the region within it required for

139   male survival identified by the exclusion of recombinants. This was achieved by examining

140   segregation of alleles from sons of the SE Asia x Moorea cross above that were mated to

141   *Wolbachia*-infected Moorea (non-suppressor) females (creating a male-informative family). Over

142   300 recombinant daughters were obtained that were used to create a linkage map of the 12

143   suppressor-linked markers. These markers covered a 41cM recombination distance and were

144   syntenic with *B. mori* (Fig. 2). The suppressor locus was localized to a region within this

145   chromosome by excluding linked loci where the SE Asia derived paternal allele was absent in one

146   or more sons (indicating the genomic region containing the SE Asia allele was not necessary for

147   male survival). Three suppressor-linked alleles, all in the +11 to +12 region, were retained in all 60

148   sons, whereas the 9 markers proximal and distal to these were excluded by the presence of one or

149   more recombinants (Fig. 2). Binomial sampling rejected the null hypothesis of no association

150   between the +11/+12 genomic region and male survival ($p=(1/2)^{60}$). Thus we posit that the

151   suppressor lies between marker C at +8 (excluded by one recombinant) and marker G at +17

152   (excluded by two recombinants).

153

154   ***The impact of suppressor spread on linked genetic variation in Samoa***

155

156   *a) Modelling a selective sweep driven by suppressor spread*

157

158   Models of selective sweep dynamics commonly utilize a fixed selective coefficient. In contrast, the

159   intensity of selection is dynamic in our system, as the benefit of rescuing a male relates to

160   population sex ratio, which depends on the frequency of male-killing *Wolbachia* and the frequency

7

161   of suppression: as the suppressor spreads, the population sex ratio shifts towards 1:1 thus reducing

162   the selective pressure for further spread. We previously modelled the spread of the suppressor in

163   this system using deterministic simulation [15]. This model tracks genotype frequency in males and

164   females separately. For each sex, zygotes are formed by random union of male and female gametes,

165   creating a genotype frequency distribution in zygotes. Male-killing then creates selection between

166   zygotic and adult phases, with any *Wolbachia*-infected males lacking the *S* suppressor gene (i.e.

167   those with genotype *ss*) being killed shortly following formation (early in development). Thus the

168   adult pool of male genotypes differs from the zygotic pool, and has a higher frequency of

169   suppression than the adult female pool. Fisherian selection is then implicit in the model, with the

170   suppressor increasing in frequency between the zygote and adult phase by virtue of being

171   overrepresented in male hosts that contribute 50% of the genetic material to the next generation.

172

173   In the Samoan population of *H. bolina*, *Wolbachia* was initially at 99% prevalence in females [4],

174   creating an extremely biased population sex ratio. Under such circumstances a novel suppressor

175   mutation will rise to 50% frequency in adult males in the first generation (all surviving F1 males are

176   heterozygous *Ss*), and to 25% frequency in the subsequent set of zygotes. Males that survive

177   *Wolbachia* infection by carrying the suppressor are now induced to express another reproductive

178   manipulation commonly employed by *Wolbachia* - cytoplasmic incompatibility (CI) against

179   uninfected females [16]. During CI, the progeny of uninfected females sired by infected males die

180   during embryogenesis, such that the presence of infected males (generated through suppressor

181   action) reduces the fitness of uninfected females relative to infected females (who experience no

182   such reduction in offspring viability following mating with infected males). Despite losing its

183   male-killing ability, the induction of CI through infected males allows *Wolbachia* to remain at or

184   near fixation. In consequence selection on the suppressor is also maintained. Indeed fixation for

185   *Wolbachia* in males and females is observed after the spread of the suppressor is present [10].

186

187   The expected dynamics of the suppressor locus in this system is given in Figure 3a, following the

188   trajectory given for $r = 0$ (zero recombination with the suppressor). We elaborated the model of

189   suppressor spread to quantify expected effects on linked loci under the assumption that the

190   suppressor and linked alleles were cost-free, and that spread was occurring through an unstructured,

191   panmictic population (see Text S1 for full details). We then modified this model to examine the

192   effect of suppressor spread on levels of association (linkage disequilibrium) between pairs of linked

193   loci, each with two alleles. Within the model, the suppressor locus can have the wild type allele *s*, or

194   the male-killing suppression allele *S*. The second locus is linked to the suppressor locus and has two

195   selectively neutral alternative alleles denoted *A* and *a*. The model tracks the change in gametic

196   frequencies from one generation to the next. There are therefore four different basic gamete types:

197   *AS*, *As*, *aS*, and *as*. Our individuals are diploid, so these four basic gamete types give nine possible

198   basic individual genotypes: *AASS*, *AaSS*, *aaSS*, *AASs*, *AaSs*, *aaSs*, *AAss*, *Aass*, and *aass*. This is

199   further complicated by the need to record whether individuals are infected or uninfected. Since

200   infection is maternally inherited we can count infection status as part of the genotype of an

201   individual, giving us a total of eighteen genotypes (the nine above, with each having infected or

202   uninfected status).

203

204   Given a set of genotype frequencies for males and females, we can derive the expected gametic

205   frequencies (incorporating recombination in males), and assuming random mating we can calculate

206   the frequencies of offspring genotypes in the next generation. We can then apply selection: we

207   assume that the infection kills all males lacking the suppressor allele (*ss*), half of the males that are

208   heterozygous (*Ss*) for the suppressor, and none of the males that are homozygous (*SS*) for the

209   suppressor. This partially dominant rescue mirrors patterns of male survival in our mapping crosses,

210 in which about half of Ss males survive. For simplicity we assume no other selective effects. This

211 process allows us to track both gametic and genotypic frequencies through time.

212

213 We iterated this process for the Samoan situation where 99% of females are infected with a fully

214 penetrant male-killer, in which males only survive in the presence of the suppressor allele, and

215 supposing that the suppressor gene initially appeared on a single infected immigrant male of

216 genotype *AASS*. The selected locus ($r = 0$) is observed to come to equilibrium within 10-15

217 generations, which is consistent with the observed rapid spread of the suppressor (Fig. 3a). Overall,

218 our panmictic model predicts the sweep would cover the entire suppressor-containing chromosome

219 (Fig. 3b), with markers at recombination distance 0.5 to the suppressor locus being swept to 35%

220 frequency. The cause of this breadth is selection in the first generation, in which effectively all

221 males in the mating pool are heterozygous for the chromosome carrying the suppressor (thus this

222 chromosome rises to an initial frequency approaching 0.25).

223

224 We further examined how the sweep would impact on the level of linkage disequilibrium (LD)

225 within the linkage group, both in terms of LD to the suppressor locus (Fig. 3c), and local LD

226 (disequilibrium between pairs of loci other than the suppressor) (Fig. 3d; Text S1). After 10

227 generations of selection, association between the suppressor locus and linked loci was weak, and at

228 20 generations it was expected to be undetectable for a sample of 50 individuals. In contrast, local

229 LD rose and fell over a longer time period. Local LD was generated by the sweep initially taking

230 locally associated variants in concert. Where the loci are closely linked to each other, the LD

231 generated was expected to be retained even by 40 generations. The extent of LD was maximized at

232 intermediate recombination distances from the suppressor locus, where swept alleles were at

233 intermediate frequency when at equilibrium.

234

235   *b) Observations of the effect of suppressor spread on patterns of genomic variation between*

236   *2001 and 2006*

237

238 We investigated the change in frequency of genetic variants associated with the spread of

239 suppression of male-killing in *H. bolina* on the island of Upolu, Samoa, through comparison of

240 allelic profiles before and after the spread of suppressor. To this end, intronic regions of the 12

241 suppressor-linked markers were sequenced for butterflies from before (2001, n=48), and after

242 (2006, n=48) the spread of the suppressor. Allele frequency in each sample was estimated using

243 PHASE and the results checked manually. The history of selection was examined directly by testing

244 for differences in allelic frequency distributions at each loci between the pre- and post-sweep

245 samples and by estimating Fst between these samples as a standardized metric of change, and

246 indirectly by the estimating LD between loci - a hallmark of recent selection. Changes were

247 compared to a control group of 9 unlinked loci located throughout the genome that would indicate

248 the presence of genome-wide effects, as opposed to suppressor linkage group specific, occurring in

249 this period.

250

251 Significant changes in the allele frequency distribution between the 2001 and 2006 population

252 samples were observed at 11 of the 12 markers along the chromosome previously identified as

253 carrying the suppressor in SE Asian butterflies (Fig. 4; all 11 with p<0.01 after sequential

254 Bonferroni correction: Fig. S1). Only the most distant marker from the suppressor (marker L)

255 showed no evidence of change. Overall, significant deviations were observed over a 25cM region

256 surrounding the location of the suppressor previously mapped in SE Asian butterflies. In contrast,

257 none of the 9 un-linked markers showed evidence of a change in allelic frequency (NS at p=0.05

258 after sequential Bonferroni correction; Fig. S2), allowing us to reject demographic factors and drift

259 as a cause of changes in allele frequency in the suppressor linkage group.

260

261   For each of the suppressor-linked loci that showed significant change from 2001-2006 we identified

262   the allele that had 'swept' alongside the suppressor. An expectation for a selective sweep is that

263   allelic distributions will be disturbed by an increase in frequency of one (or sometimes two) alleles

264   initially associated with the target of selection, which would be paralleled in a co-ordinated decline

265   in frequency of the other alleles. Because decline is spread across multiple alleles, the expectation is

266   that a swept allele should be the greatest contributor to heterogeneity between allelic frequency

267   distributions, and thus identifiable by the largest standardized residual in heterogeneity tests. We

268   performed this analysis of residuals at 10 of the loci where heterogeneity between samples was

269   observed (one locus was not suitable for analysis in this manner, as there were only two allelic

270   variants, thus by definition each allele contributes equally to heterogeneity). At each of the 10 loci,

271   a single allele increasing in frequency from 2001 to 2006 contributed the largest residual to

272   heterogeneity analysis, and thus can be regarded as the swept allele. However in 3 cases (loci B, D

273   & G), removal of this allele did not restore homogeneity, and a second allele that increases in

274   frequency can additionally be identified as contributing to the heterogeneity observed, albeit with a

275   lower magnitude of frequency change (swept alleles annotated with arrows in Fig. 4).

276

277   We also calculated the Fst statistic for each locus as a standardized magnitude of difference

278   between the 2001 and 2006 population samples (Fig. 5). Fst differentiation was highest in the 5cM

279   region in which the suppressor had been previously located in SE Asian butterflies (Fst=0.2-0.3),

280   and lower, but significant, differentiation was found over the 25cM region surrounding the locus

281   ($p<0.01$ at all loci after sequential Bonferroni correction), making this selective sweep the broadest

282   observed to date in nature. Fst differentiation was either absent or present at a very low level

283   (Fst<0.03) in the group of 9 unlinked markers (Fig. S3).

284

285   The pattern of LD also reflected an episode of selection in this genomic region. Our model above

286   predicted that LD between the swept loci and the target of selection (global LD) would exist only in

287 the very early phases of the sweep (10-20 generations), but that the sweep would create multiple

288 local associations between closely linked loci that would be retained over longer periods (local LD)

289 (Fig. 3c, 3d). In accord with this, there was little evidence of LD between loci in the 2001 pre-

290 suppressor sweep sample, but strong associations between variants at closely linked loci (r=0.01-

291 0.02) in the 2006 sample (Fig. 6).

292

293 Our data indicate that the genomic region identified in SE Asian butterflies as carrying a suppressor

294 locus was also the focus of selection in the selective sweep in Samoa. This observation has two

295 possible explanations. First, the suppressor mutation may have been introduced into Samoa by

296 migration. Given the suppressor is absent in the nearest island groups, American Samoa and Fiji,

297 suppressor introduction would be associated with a long distant migrant. Second, the genomic

298 region may represent a hotspot for suppressor mutation, derived independently in Samoa by *de novo*

299 mutation.

300

301 The presence of novel swept alleles at linked loci indicates that migration is the most parsimonious

302 explanation for suppressor origin. Variants not present in the 2001 sample were observed to be the

303 main 'swept' allele at 4 of the 11 loci at which significant change was detected (indicated with

304 green arrows in Fig. 4). At two of these loci (A & I), the invading allele was defined by a SNP

305 being not present in the 2001 sample, whereas the other two alleles represented different

306 combinations of existing SNPs. The four loci were in three genomic locations spaced over 17cM

307 and showed no evidence of linkage disequilbrium (association) in the 2001 pre-sweep sample, and

308 thus they can be treated as independent (Fig. 6). We believe it is very unlikely that all four alleles

309 existed in the 2001 population but were not sampled. We reason that derivation of alleles selected

310 by correlation to a *de novo* suppressor mutation would occur in proportion to their frequency in the

311 pre-sweep population. There is an upper confidence limit for the frequency of alleles absent in the

312 2001 sample of 0.0307 (binomial sampling n=96 alleles, 95% confidence interval). Taking the

13

313   conservative assumption that each absent allele existed at the upper confidence level for frequency,

314   four or more unsampled alleles being present in a sample of 11 as targets of selection occurs with a

315   chance of p=0.00025 (calculated from binomial sampling distribution:

316     $\sum_{k=4}^{11}\binom{11}{k}0.0307^k(1-0.0307)^{11-k}=0.00025$, where each locus has a chance of 0.0307 of having a

317   previously unsampled allele, and obtaining four or more such alleles from 11).

318

319   Thus the presence of four novel linked variants argues against a model of *de novo* mutation to

320   suppression. In addition, two of the three secondary 'swept' alleles (those that contributed

321   significantly to locus heterogeneity between the 2001 and 2006 samples, after the main swept allele

322   had been removed) were also novel in 2006 (markers B & D - denoted by dotted green arrows in

323   Fig. 4). We thus conclude a migratory source for the suppressor represents a more parsimonious

324   explanation for the origin of suppression, although definitive proof of this awaits fine-scale

325   mapping and characterization of the suppressor mutation itself both within Samoa and from

326   possible source populations (e.g. SE Asia).

327

328   Notwithstanding the broad sweep observed, changes across the suppressor chromosome were not as

329   profound as our model predicted. It is likely that the assumption of panmictic population structure

330   within the model accounts for this difference. In natural populations, the suppressor mutation will

331   occur initially at one geographical location, and the mutation would then spread away from this

332   location each generation in a wave of advance. This spatial spread of suppression was observed in

333   Savai'i, the neighbouring island to Upolu, and took at least one year (10 generations) to complete

334   [10]. Each generation of spatial advance would have seen a less broad local sweep, as association of

335   loci to the suppressor locus would have already been eroded by recombination in previous

336   generations (see model output, Fig. 2c). Markers that are only weakly linked (e.g. c=0.25) would

337   therefore only be swept in the immediate vicinity of the initial suppressor mutation, and not in the

338   remainder of the species range, diluting the impact seen at these loci.

339

340   *c) Observations of the pattern of genetic variation at suppressor-linked loci post 2006*

341

342   In order to investigate the longer term impact of the sweep, or to identify its terminus, we also

343   analysed the pattern of variation within the linkage group for a further population sample collected

344   in 2010 (*c.* 32 generations after the 2006 sample). Local LD was still observed, but, as expected

345   from continued recombinational erosion, the extent of LD was reduced (Fig. 6c). In terms of allelic

346   profile, the pattern observed was heterogeneous (Fig. 4; Fig. 5). At six loci (including the three in

347   the direct vicinity of the region containing the suppressor), allele frequencies in the 2010 sample

348   were not significantly different from the 2006 sample. Differentiation of the 2010 to the 2001

349   samples, as measured by Fst, was equivalent to that previously observed in 2006. The allelic

350   frequency distributions at these loci are at equilibrium, and from these we conclude the sweep had

351   effectively ended. However, allele frequency distributions differed between 2006 and 2010 samples

352   at 5 loci across two genomic regions, one proximal to that carrying the suppressor, and one distal to

353   it. For these, Fst differentiation to the 2001 sample was reduced, and significant differentiation to

354   the 2006 sample was also observed (Fig. 5). In all cases the differentiation is caused by loss (or

355   reduction in frequency) of the major allele that had been swept between 2001 and 2006. Our

356   sampling was geographically broad for both 2006 and 2010 samples, leading us to reject population

357   substructure as a cause of allele frequency heterogeneity.

358

359   We finally examined whether the loss of originally swept suppressor-linked alleles by 2010 could

360   be explained by genetic drift. Using the model of Kimura ([17], equation 7) we ascertained the

361   effective population size at which there is a reasonable chance an allele present in 2006 would be

362   absent in 2010. We conducted this for locus G, where the swept allele was at a frequency of 0.22

15

363   (n=94) in 2006 and absent in 2010. Setting the time elapsed between samples conservatively at 40

364   generations (4 years, 10 generations per year based on egg-mature adult period of 36 days), we

365   estimated that loss would occur on 1% of occasions with $N_e = 180$, and on 5% of occasions with

366   N_e=122. Given the size of Upolu, Samoa, and the ease of collecting adult butterflies (our samples of

367   48 were collected in two days from three sampling points, and did not involve sampling

368   pupae/larvae), we consider it likely that N_e is considerably larger than this, and thus conclude that

369   drift was unlikely to cause the loss of material observed. Furthermore, we note that loss of

370   introgressed material occurred at two genomic locations that were not in LD with each other, again

371   making chance sampling an unlikely source of the loss of material. Rather, we hypothesize that

372   purifying selection against introgressed material is the most likely reason for the loss of swept

373   alleles.

374

375   ***The evolution of suppression in Samoa***

376

377   Our results lead to the following working hypothesis for the evolution of suppression in Samoa. In

378   2001 the population did not carry the suppressor; nearly all individuals were infected with the male-

379   killer, and the population sex ratio was very female biased, with 100 females per male. Between

380   2001 and 2005, the suppressor allele was introduced by immigration, consistent with selection

381   occurring in the same genomic region identified as required for male survival in SE Asian

382   butterflies, and the introduction of several novel variants across the swept region. The suppressor

383   was then under very strong selection, as shown by our theoretical model and the rate of change of

384   sex ratio observed in nature [10]: in the first generation after arrival of the immigrant effectively all

385   males in the local mating pool would carry a copy of the suppressor, creating a population

386   frequency of 25%. This intense initial selection was sufficient to produce significant introgression

387   across at least a 25cM region surrounding the locus under selection, which represents the widest

388   genomic region selected during a sweep in a natural population. The sweep had reached equilibrium

16

389 by 2006. Subsequently, some of the linked genomic regions that had been introduced along with the

390 suppressor declined in frequency, most likely because the introduced alleles were deleterious in the

391 novel Samoan background.

392

393 The intensity of selection associated with sex ratio distorting elements may be biologically

394 important because it leads to the invasion of variants that would otherwise be deleterious. The

395 suppressor mutation itself may be highly deleterious in the absence of *Wolbachia* infection, yet

396 invade and become an essential component because it is necessary for male host survival, and its

397 spread does not reduce the frequency of the symbiont (thus it remains required for male survival). It

398 has been widely conjectured that the evolution of sex determination systems might occur in

399 response to the presence of sex ratio distorting microbes, and our data indicates that dramatic

400 changes with associated pleiotropic deleterious effects may be nevertheless be favoured if they

401 rescue males. A pressing area for research is to establish the actual nature of the suppressor

402 mutation (e.g. whether it is part of the sex determination cascade), whether there is a cost of

403 carrying a suppressor in the absence of its benefit from rescuing males, and whether any

404 compensatory evolution has occurred where the suppressor has been present for a longer period.

405

406 Beyond the locus under selection, linked variants may also be deleterious. This is one explanation

407 for the gain followed by loss of introgressed material. Our hypothesis is that some introgressed

408 alleles were deleterious on the Samoan genetic background, associated with negative epistasis

409 (Dobzhansky-Muller incompatability) generated during isolation on Samoa. Selection against

410 linked deleterious recessive alleles provides an alternative explanation, although the complete loss

411 observed for some loci suggests that selection against the material is maintained when rare, arguing

412 against recessive effects. Whatever the precise cause of the loss of introgressed material, an

413 important conclusion from our data is that the initial strength of selection would be seriously

414 underestimated from genetic variability data obtained after the final phase of the sweep alone (e.g.

17

415   2010 data). Thus, for cases of strong selection over a wide region, caution is needed when

416   estimating the strength of selection from post-sweep genetic data alone.

417

418   Finally, our data highlight the profound loss of evolutionary capacity associated with isolation. In

419   Samoa, at least 100 years of extreme sex ratio bias was resolved by a migratory origin of the

420   suppressor that likely involved individual movement over very long distances across the Pacific,

421   rather than *in situ* mutation. One question that still requires answer is 'why 100 years'. This

422   observation should be considered in concert with our wider knowledge of suppressor spread in this

423   system. First, suppression of male-killing was known in the Phillipines in 1890, but was not

424   observed in neighbouring Borneo (despite presence of a high prevalence male-killing *Wolbachia*)

425   until after 1970 [18]. Suppression was also absent in Hong Kong at this time [18], but present in

426   2005 [9]. Indeed, by 2005, suppression of male-killing was observed widely across SE Asia [9].

427   Thus, there was a larger 'front' of suppressor-carrying populations building in SE Asia, making

428   arrival of a migrant much more likely after 1970. Evolutionary biologists commonly regard island

429   isolation as a driver of novelty, both in terms of phenotypic traits and endemism. Our study makes it

430   clear that small isolated populations may also be constrained in their response to novel evolutionary

431   challenges. This may be particularly likely for host-parasite symbiosis where there are precise

432   molecular interactions determining parasite persistence and virulence, and few feasible mutations

433   available to provide resistance.

434

435   **Materials and methods**

436

437   *Developing a genome-wide marker set for H. bolina*

438

439   We utilized high-throughput sequencing of the transcriptome of *H. bolina* to obtain coding

440   sequence from multiple loci across the genome. Following total RNA extraction from 10 *H. bolina*

441   pupae, mRNA library construction and sequencing using the Roche 454 sequencing platform

442   (http://www.454.com), 450 bp reads were *de novo* assembled into contigs using the Newbler

443   assembler to create the first set of Expressed Sequence Tags (EST) for *H. bolina*.

444

445   In the absence of any annotated genome or transcriptome for *H. bolina*, the moth *Bombyx mori* was

446   used as a proxy reference genome, this being the only available resource for Lepidoptera at the time

447   of the study. There is a high level of synteny of gene location in the Lepidoptera [19] allowing a

448   targeted gene approach, in which several genes could be selected from each chromosome across the

449   genome. Coding sequence of highly conserved genes such as ribosomal proteins and housekeeping

450   genes from *B. mori* were targeted and then retrieved from NCBI (http://www.ncbi.nlm.nih.gov). To

451   determine putative *H. bolina* orthologs a local tBLASTx was then performed against the *H. bolina*

452   EST set. Only genes that returned a single tBLASTx hit were included, reducing the likelihood of

453   the inclusion of paralogs in our marker set. The orthologous *H. bolina* contigs were then translated

454   into amino acid sequences using the ExPASY online tool (http://web.expasy.org/translate), with the

455   sequence lacking mid-sequence stop codons chosen as the most likely translation. In a final test for

456   paralogs, a reciprocal BLAST was performed of coding sequence from the orthologous *H. bolina*

457   contigs as queries against the *B. mori* genome using the INPARANOID8 search tool ([20];

458   http://inparanoid.sbc.su.se/).

459

19

460   Intronic regions were targeted for marker development, as they are likely to have a higher degree of

461   nucleotide diversity. Again, conservation of synteny in Lepidoptera genome organisation allowed

462   the intron/exon boundaries in *H. bolina* genes to be inferred using the *B. mori* genome. Through

463   tBLASTx analysis of the *B. mori* coding sequence of the targeted gene against the *B. mori* WGS

464   (Whole Genome Shotgun contigs) database in NCBI, exonic regions were identified (as only these

465   regions will align). The translated orthologous *H. bolina* contig and the corresponding *B. mori*

466   amino acid sequence were aligned using ClustalW [21] and the position of the intron/exon

467   boundaries subsequently located.

468

469   Once intron/exon boundaries were identified in *B. mori* genes, and extrapolated to the *H. bolina*

470   orthologous sequences, primers were designed for *H. bolina* that spanned introns of size 500-

471   1000bp (*Bombyx* size approximation). This size range was chosen to enable successful

472   amplification of the intronic region during PCR. Marker optimisation was performed using three

473   test *H. bolina* samples and successful PCR products were sequenced using Sanger technology.

474   From this, 46 markers from 23 chromosomes were chosen that gave clean sequences, with a

475   preference for loci that had 5 or more single nucleotide polymorphisms (SNPs).

476

477   ***Mapping the suppressor linkage group***

478

479   In order to investigate the genetic architecture of male-killing suppression in *H. bolina* and

480   determine markers in linkage with the suppressor locus we crossed females of a butterfly population

481   (the Philippines) that were *Wolbachia*-infected and homozygous for the male-killing suppressor

482   allele (***SS***) to males from a *Wolbachia*-infected population (Moorea, French Polynesia) that lacked

483   the suppressor (*ss*), to create suppressor-heterozygous *Wolbachia*-infected offspring (***S****s*) (Fig. 1).

484

485   Recombination does not occur during female meiosis in the Lepidoptera [22], permitting the

486   progeny of **S**s females to be used to identify the linkage group (SLG, Suppressor Linkage group) in

487   which the dominant suppressor allele was carried. To this end, **S**s females were crossed with ss

488   males to produce the female-informative families. For inclusion in the SLG, markers linked to the

489   suppressor locus are characterized by being present in all surviving sons of the **S**s heterozygous

490   mother, rather than the 50% expectation from Mendelian segregation with random survival. Initially

491   each marker was sequenced in the F1 parents (**S**s female x ss male). In each case, SNPs were

492   chosen that were heterozygous in the female and homozygous in the male – following the presumed

493   pattern of the suppressor. These same SNPs were then scored in 16 male and 8 female F2 progeny.

494   Once a marker had been found that was present in half of the daughters (following Mendelian

495   inheritance) but all of the sons (for a son to survive it must have at least one copy of the suppressor,

496   and hence linked marker allele), further markers were developed for that same chromosome based

497   on synteny with *B. mori*. A final suite of 12 markers that produced clean sequence and that spanned

498   the suppressor-associated chromosome were developed to form the SLG.

499

500   Recombination does occur in male *H. bolina*, and thus crosses of **S**s males to ss females (the male-

501   informative families) allow a) mapping of genetic markers within a chromosome relative to each

502   other and b) mapping of the suppressor within the linkage group, in terms of a region of the

503   chromosome that is always present in surviving sons. To this end, the 12 linked markers were

504   sequenced in the female F2 (n = 307) from one male informative cross (**S**s male x ss female) and a

505   linkage map created using JoinMap (version 3.0) [23]. To place the suppressor locus within the map

506   F2 males (n = 60) from this cross were analysed using the same 12 markers. Absence of

507   recombinants in a core subset of markers, flanked by markers with an increasing numbers of

508   recombinants, indicated the position of the suppressor locus (Fig. 2).

509

510

21

511   ***Assessing the effect of suppressor spread on genomic variation***

512

513   A population sample of butterflies from three time points (2001: n=48, 2006: n=48, 2010: n=46)

514   were collected from one region on the Samoan island of Upolu. For each individual, DNA was

515   extracted using the Qiagen DNEasy kit, and the suite of 12 suppressor-linked markers amplified

516   using PCR. Following Sanger sequencing of the amplicons through both strands, the resultant

517   marker sequences were alignment in Codoncode (http://www.codoncode.com/). SNPs present

518   within and between the population samples were then identified and scored for each individual

519   butterfly. Using the SNP data, the alleles present at each marker in each population sample were

520   estimated using the haplotype reconstruction software PHASE (version 2.1 [24,25]) with 1000

521   iterations, a thinning interval of 100 and 1000 burn-in iterations. Allele frequencies at each marker

522   for each time group could then be calculated and compared. Output was also examined by eye, with

523   alleles identified first where there was no ambiguity (either homozygous, or a single SNP separating

524   into two defined alleles). Thereafter, alleles were assumed identical to those already identified

525   where possible. The low allelic diversity meant this visual analysis produced very similar result to

526   PHASE output, which can thus be considered robust.

527

528   Patterns of differentiation were estimated using GENEPOP [26]. First, heterogeneity of allelic

529   frequency distributions between pairs of time points was estimated using a G test based on allelic

530   frequency distribution. Where allele distributions were heterogeneous, we ascertained the allele

531   whose frequency change made the greatest contribution to heterogeneity as that with the largest

532   standardized residual within the heterogeneity test. This allele was then removed (it was an allele

533   increasing in frequency in each case), and the data retested to ascertain if the population samples

534   were then homogeneous, or whether there was evidence for a second allele that changed in

535   frequency (a second allele was identified in three cases). We additionally used Fst standardized

536   population genetic differentiation to quantify the magnitude of change between allelic frequency

22

537   distributions between the two samples. In each case, the rare individuals where sequence could not

538   be obtained for particular alleles, or not inferred accurately, were coded as missing information.

539

540   Nine unlinked markers, each situated on a different chromosome, were also sequenced for the 2001

541   and 2006 population samples to investigate the degree to which changes were observed in the wider

542   genome and as a control for demographic effects. These were tested for the presence of

543   heterogeneity between time points using a G test based on allelic frequency distributions, and for

544   differentiation using the Fst statistic.

545

546   We additionally analysed evidence for alteration in the pattern of linkage disequilibrium, again

547   using GENEPOP. The significance of LD between all possible combinations of loci was tested in

548   the 2001 and 2006 samples separately. We do not report the magnitude of LD, as this is not a

549   standardized measure, being dependent on the allelic frequency distribution at each locus.

550

555

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614

615   **Figure legends**

616

617   **Figure 1. Mapping of the *Hypolimnas bolina* genomic region surrounding the suppressor of**

618   **male-killing.**

619   A *Wolbachia* infected (denoted by 'w') female that was homozygous for the suppressor allele (**_SS_**)

620   was crossed to a uninfected male that did not carry this allele (*ss*). To produce a female-informative

621   family, *Wolbachia*-infected heterozygous daughters (**_S_***s*) from this pairing were in turn crossed to

622   uninfected males lacking the suppressor. Because there is no recombination in female Lepidoptera,

623   male survival is associated with inheritance of the linkage group carrying the suppressor, and

624   suppressor-linked loci can be identified as those present in all surviving F2 sons (those marked with

625   a cross die) but only 50% of F2 daughters. To produce a male-informative family, *Wolbachia*-

626   infected heterozygous sons (**_S_***s*) from the original parental cross were crossed to infected females

627   lacking the suppressor. Using this cross, members of the suppressor-associated linkage group were

628   mapped relative to each other through the pattern of recombination in the F2 daughters. The

629   location of the suppressor was ascertained as the genomic region that was present in all surviving

630   F2 sons.

631

632   **Figure 2. Recombinational map of the *H. bolina* chromosome carrying the suppressor locus.**

633   Depiction of the suppressor linkage group (SLG), with the 12 linked markers A-L. The genomic

634   region containing the suppressor locus is highlighted in green. Numbers represent distance from the

635   distal marker in cM as estimated using JoinMap. The broadly syntenic *B. mori* chromosome 25 is

636   given for reference.

637

638   **Figure 3. Predicted impact of selective sweep on the frequency and association of linked**

639   **variants**. 3a) Change in the frequency of initially associated linked variants over time at different

640   recombination distances, 3b) Equilibrium frequency of initially associated linked variants at

26

641 different recombination distances, 3c) Linkage disequilibrium between loci at different

642 recombination distance from suppressor after 5, 10 and 20 generations of selection, 3d) Local

643 linkage disequilibrium between alleles with recombination probability of 0.01 at different

644 recombination distances from the suppressor. The four curves show the situation after 5, 10, 20, and

645 40 generations (increasing lightness of colour corresponding to greater number of generations).

646

647 **Figure 4: Allelic frequency distributions across the linkage group carrying the suppressor in**

648 **2001, 2006 & 2010.** The major allelic variant for each locus (A-L) that has increased in frequency

649 during the sweep is indicated with a solid arrow, while the secondary allele that increases (in 3

650 cases) is indicated with a dotted arrow. Where the allele is novel in 2006/2010 the arrow is green.

651 Results of statistical comparison presented in Fig. S1. Effective allele numbers (A_E) are indicated

652 on each graph.

653

654 **Figure 5: Fst standardized population genetic differentiation between samples from different**

655 **time points for each locus along the chromosome carrying the suppressor.** Blue: 2001-2006;

656 Red: 2001-2010; Green: 2006-2010. Statistically significant deviations from Fst=0 given by ***

657 (p<0.001), ** (p<0.01) * (p<0.05) uncorrected for multiple tests.

658

659 **Figure 6: The pattern of linkage disequilibrium (association) between loci observed in 2001**

660 **(pre-sweep), 2006 (immediately post-sweep) & 2010 (4 years post sweep) population samples.**

661 Pattern of linkage disequilibrium (LD) between suppressor-linked loci (A-L) for a) 2001 (pre-

662 sweep), b) 2006 (immediately post-sweep), and c) 2010 (4 years post-sweep). Significance denoted

663 by colour: deep red – significant LD as measured at p<0.001; mid red – significant LD as measured

664 at p<0.01; pink – significant LD as measured at p<0.05, all uncorrected for multiple tests. Depiction

665 of suppressor linkage group given for reference.

666

667

668 **Supporting Information Figure Legends**

669

670 **Figure S1. Results of statistical testing for genotypic differentiation between population**

671 **samples at loci in the linkage group carrying the suppressor.** Significance denoted by colour:

672 deep red – significant differentiation as measured at p<0.001; mid red – significant differentiation

673 as measured at p<0.01; pink – significant differentiation as measured at p<0.05, all uncorrected for

674 multiple tests.

675

676 **Figure S2. Allelic frequency profiles of markers unlinked to the suppressor.** Each of the 9 loci

677 (a to i) is situated within a different chromosome in the *H. bolina* genome, derived from *Bombyx*

678 *mori* genomic annotations. Allele frequency changes between 2001 (red) and 2006 (blue) at all 9

679 loci were not significant (Chi Square heterogeneity test; p>0.05, Bonferroni corrected).
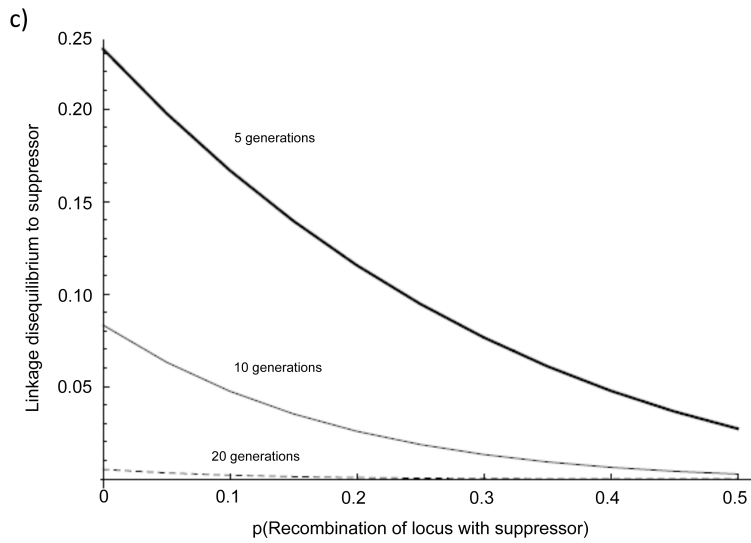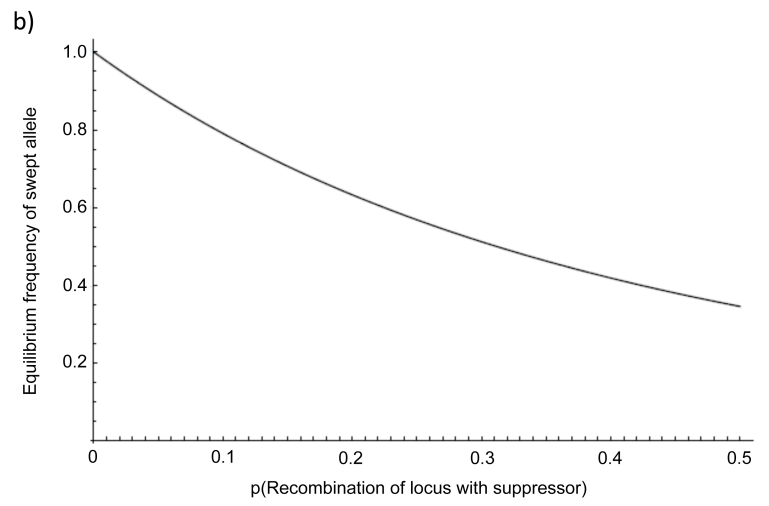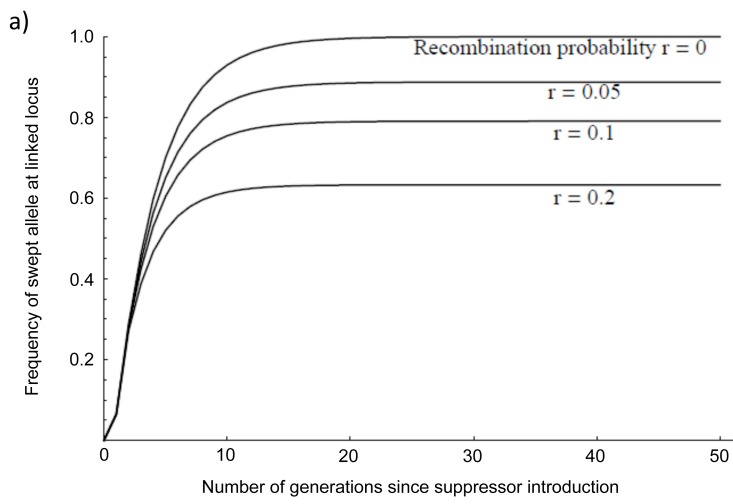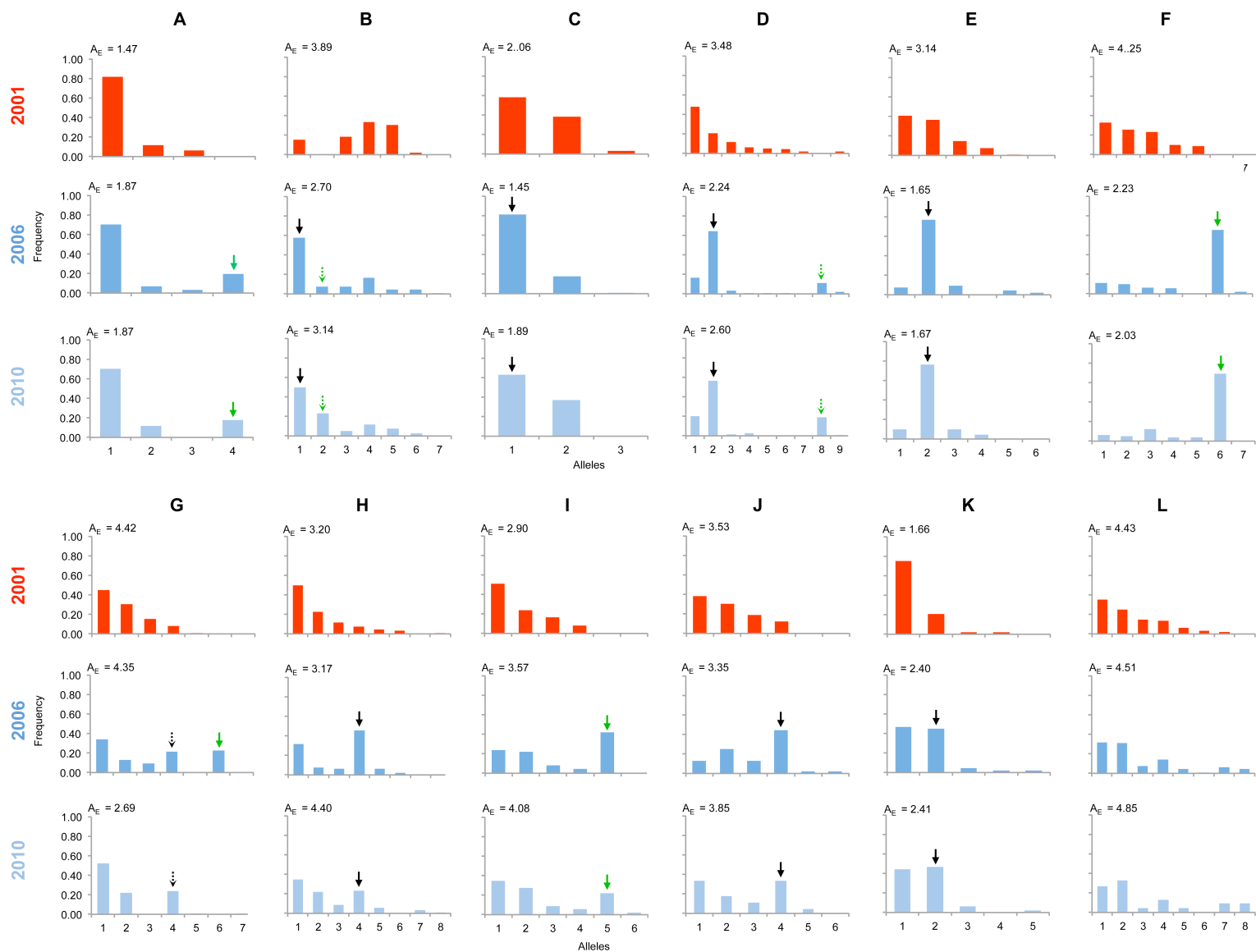
680

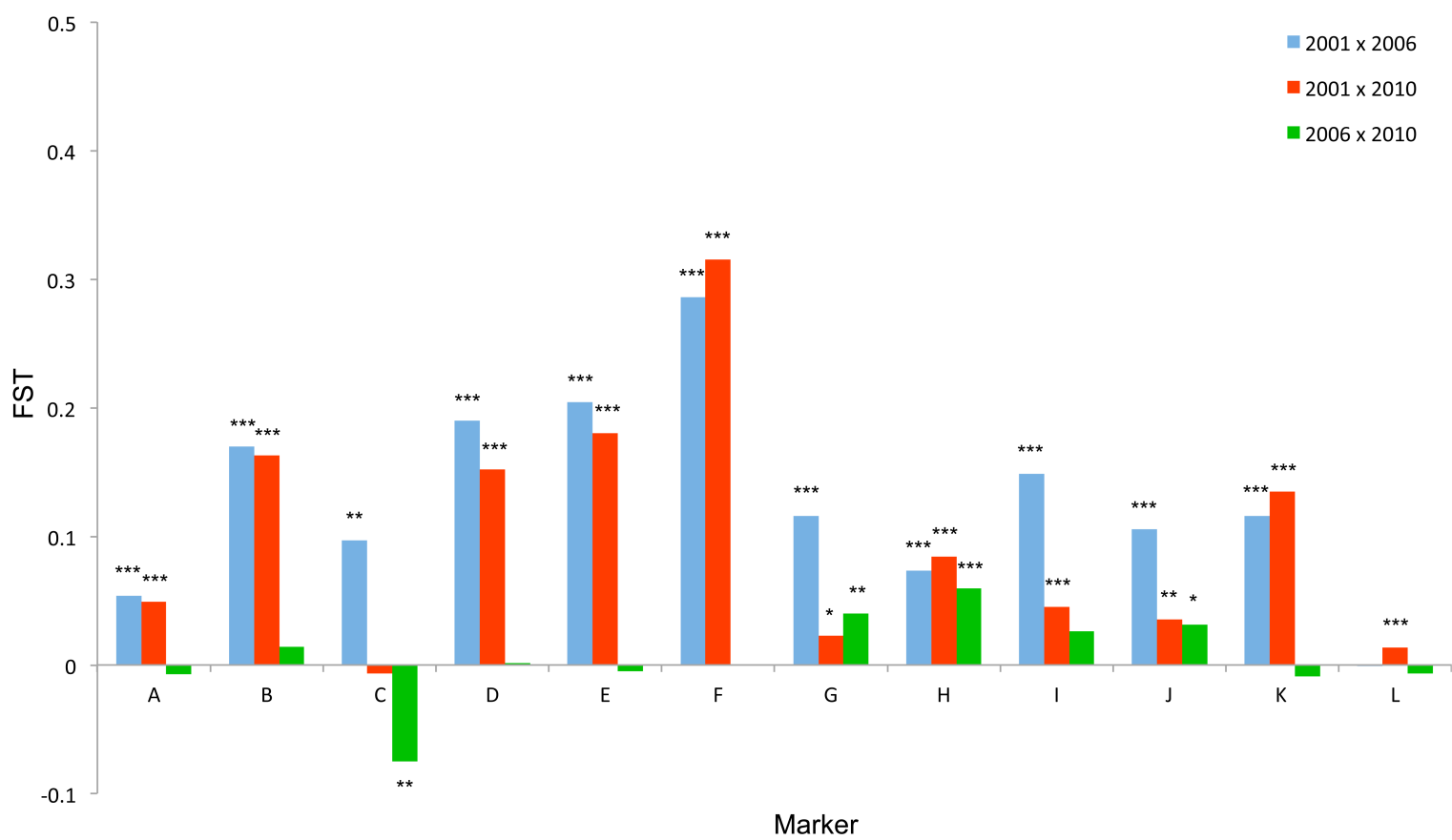681 **Figure S3. Fst analysis of differentiation between 2001 and 2006 population samples at the**

682 **nine unlinked loci.**

no recombination
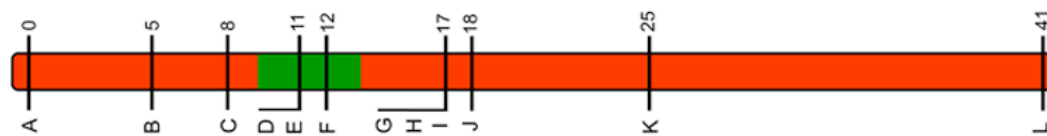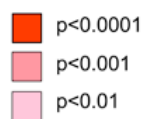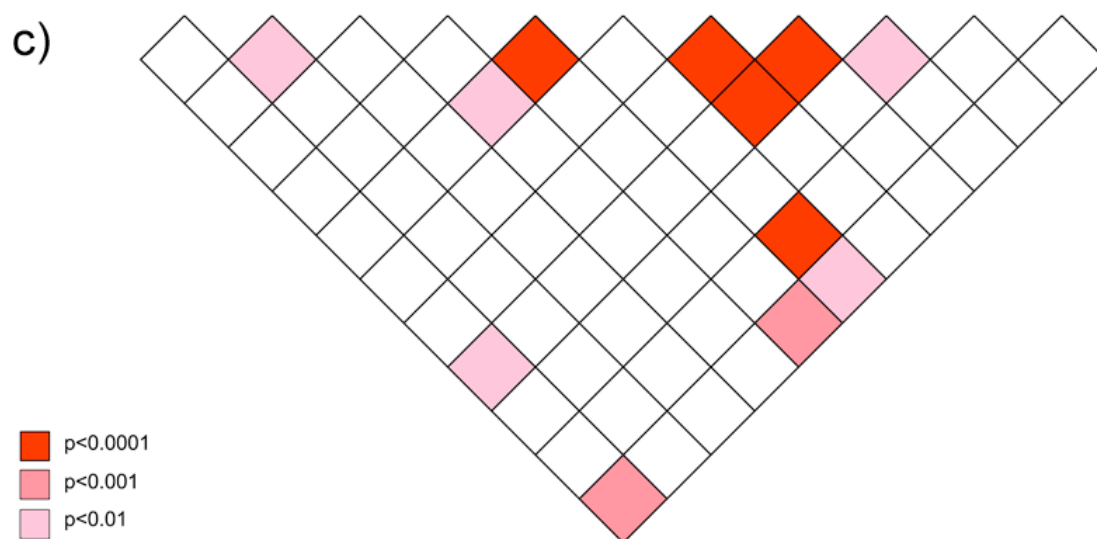
recombination

H. bolina
SLG

B. mori
Chr. 25

0 — A
5 — B
8 — C
D
11 — E
12 — F
G
H
17 — I
18 — J
25 — K
41 — L

a)

Frequency of swept allele at linked locus

Recombination probability r = 0

r = 0.05

r = 0.1

r = 0.2

Number of generations since suppressor introduction

b)

Equilibrium frequency of swept allele

p(Recombination of locus with suppressor)

c)

Linkage disequilibrium to suppressor

5 generations

10 generations

20 generations

p(Recombination of locus with suppressor)

d)

Linkage disequilibrium

p(Recombination of locus with suppressor)

	A	B	C	D	E	F	G	H	I	J	K	L
2001x2006												ns
2001x2010			ns									
2006x2010	ns	ns		ns	ns	ns		ns			ns	ns

ns	Non significant
	p<0.05
	p<0.001
	p<0.0001
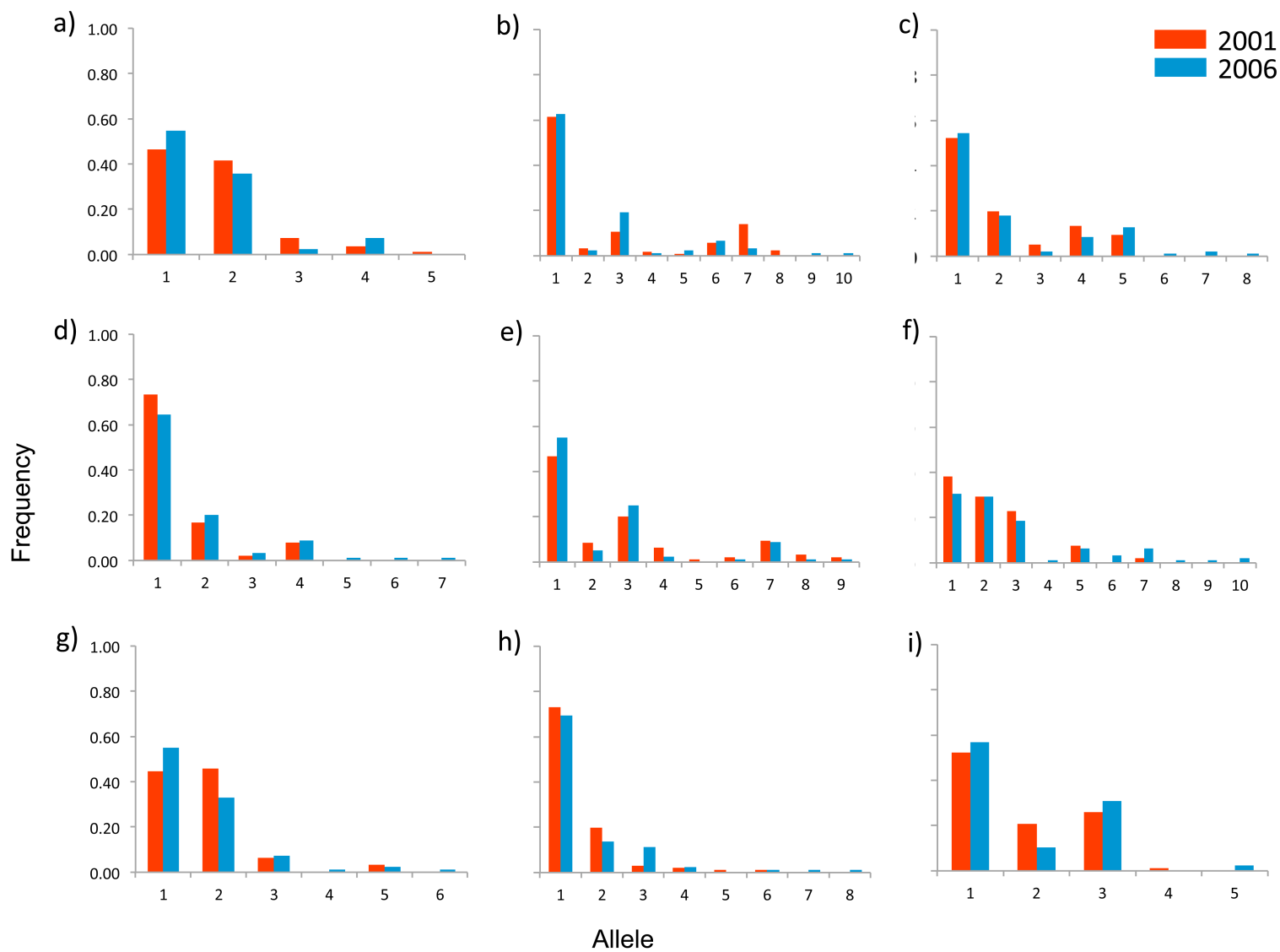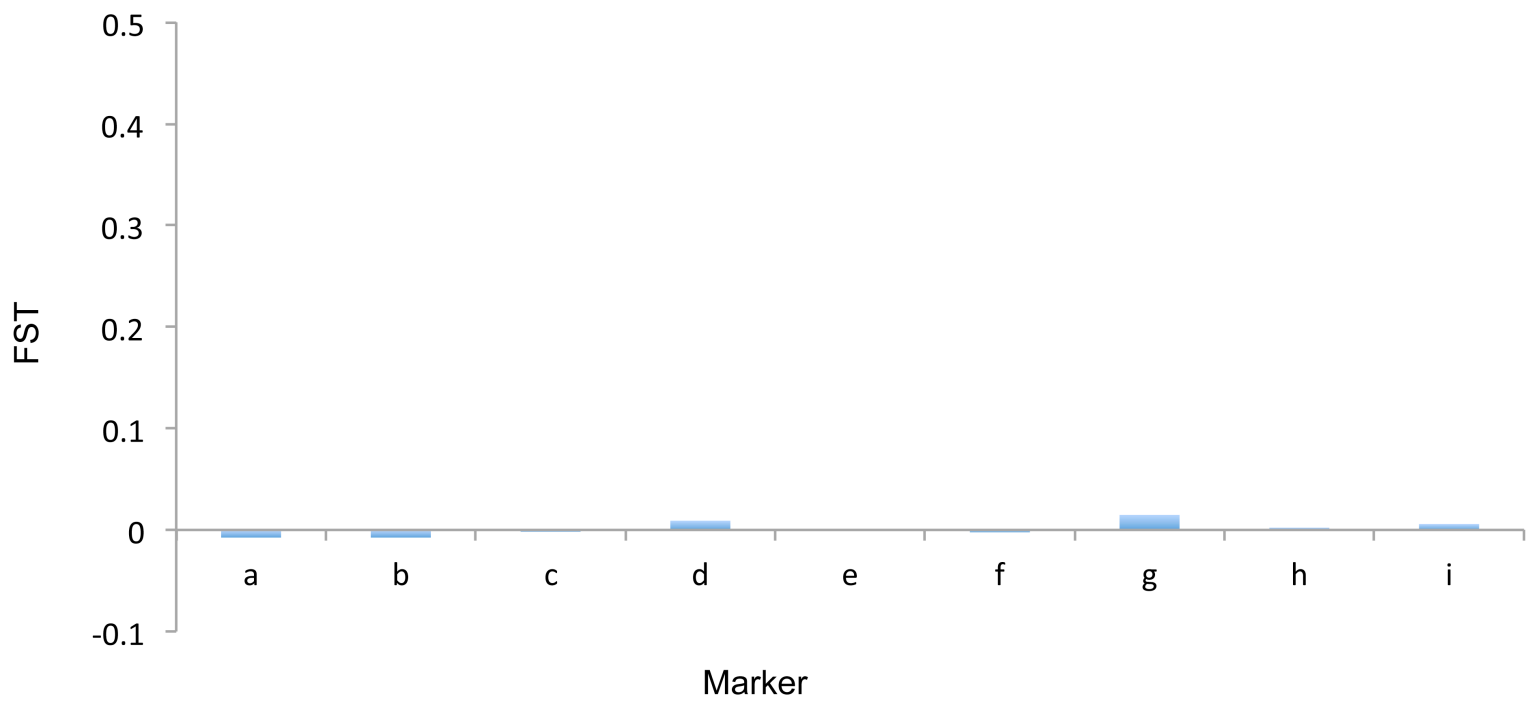
Text S1: Modelling the spread of the suppressor

The model

We coded the model in Mathematica.

We model two linked loci, each with two alleles. The first locus can have the wildtype allele *s*, or the male-killing suppression gene *S*. The second locus is linked to the first, and has two selectively neutral alternative alleles denoted *A* and *a*. The model tracks the change in gametic frequencies from one generation to the next. There are four different basic gamete types: *AS*, *As*, *aS*, and *as*. Our individuals are diploid, so these four basic gamete types give nine possible basic individual genotypes: *AASS*, *AaSS*, *aaSS*, *AASs*, *AaSs*, *aaSs*, *AAss*, *Aass*, and *aass*.

The population is infected with *Wolbachia* at a frequency *J*. The infection is vertically transferred from mothers to offspring with 100% efficiency. Thus all of the offspring of an infected mother are infected, and none of the offspring of an uninfected mother are infected. Since there is this difference between males and females, we have to distinguish between male and female gametes. We also have to add infection status to the gametic genotypes to give a total of sixteen gamete types: four basic types, each from a male or female, and either infected or uninfected. Infection status and sex are also added to the individual genotypes to give thirty-six possible genotypes (the nine genotypes from above can be either infected or uninfected, and male or female). Note that for notational ease we still refer to these as "genotypes" although infection status is not a property of the individual's genome.

The life cycle consists of mating, then selection. The gametic frequencies we begin with give rise to offspring genotypic frequencies. These offspring frequencies undergo selection, and thus result in adult genotypic frequencies. The adults produce gametes, which give us the gametic frequencies for the next generation. Along the way we can record the adult genotypic frequencies and consequently make predictions about the allelic frequencies observed in the real world.

In our model it is not generally the case that there are equal numbers of males and females, because *Wolbachia* affects the two differently. However, there are always equal numbers of male and female gametes (because each mating involves exactly one male and one female). Therefore the female gamete frequencies sum to one, and so do the male gamete frequencies.

Mating is assumed to be at random, and the mating step in our model consists of the transformation of the gametic frequencies into offspring genotypic frequencies. Given a female gamete at frequency *f*, and a male gamete at frequency *m*, the frequency of offspring resulting from combining these two gamete types will be the product of their frequencies *fm*. An added complication arises due to cytoplasmic incompatibility (CI). This is when mating between infected males and uninfected females result in fewer offspring than other matings. For simplicity, we assume that CI is total, so that fusions between infected male gametes and uninfected female gametes result in no offspring. We then renormalise the offspring frequencies appropriately so that they still sum to one for both males and females.

Once we have the offspring genotypes, selection occurs, transforming the offspring genotypic frequencies into adult genotypic frequencies. In our model, the only element of selection is male-killing: any infected males lacking the *S* suppressor gene (i.e. those with genotype *ss*) are killed by the *Wolbachia*. Males heterozygous at the suppressor locus (i.e. with the genotype *Ss*) are killed by their infection 50% of the time. Males homozygous for the suppressor (i.e. those with genotype *SS*) are not killed. For simplicity we assume no other selective effects. Notably this means that we are modelling the situation in which neither the suppressor nor the linked allele impose costs on their bearers.

Because females are unaffected by *Wolbachia*, there are now more females than males in our population. This will affect the observed allelic frequencies, since the allelic frequencies in males and females will differ (*s* genes, for example, will be more common in females because they are selectively neutral to a female, while males bearing *s* genes are more likely to die through male-killing). To account for this fact we renormalise the post-selection genotypic frequencies so that the sum of both male and female genotypic frequencies is one. This gives us the adult genotypic frequencies. From this data we get the model's predictions for observed frequency of *A* and *S* alleles.

To complete the generation, we finally transform the adult genotypic frequencies into gametic frequencies. This is trivial in the case of most of the genotypes. However, for genotypes *AaSs*, things are more complicated. This is because *AaSs* individuals could have been formed by *AS* x *as* crosses, or by *As* x *aS* crosses. Denote the probability that a randomly-chosen *AaSs* individual was formed by an *AS* x *as* cross by μ, and the probability of recombination between the two loci of interest by r. Then the frequency of gamete types from infected *AaSs* individuals is

AS: ½($\mu(1-r)+(1-\mu)r$)
aS: ½($1-\mu(1-r)-(1-\mu)r$)
As: ½($1-\mu(1-r)-(1-\mu)r$)
as: ½($\mu(1-r)+(1-\mu)r$).

If we denote the probability that a randomly-chosen uninfected *AaSs* individual was formed by an *AS* x *as* cross by ν we can produce similar frequencies. It remains only to find μ and ν. But since we know the previous generation's gamete frequencies this is trivial.

In the initial population of size *N* we suppose that the *A* and *S* alleles are absent. With a proportion *J* of the population infected with *Wolbachia*, the gamete types and frequencies in the population are therefore infected female *as* (frequency *J*), uninfected female *as* (frequency $1 - J$), and uninfected male *as* (frequency 1). At this point the sex ratio is $(1 - J)/(2 - J)$. We then introduce an immigrant of known sex and genotype into the population. To do this, we multiply the male gamete frequencies by $N(1 - J)/(2 - J)$, and the female gamete frequencies by $N/(2 - J)$ to give a "gamete mass" measurement. Then, given the immigrant's genotype we get the probability that it produces a gamete of each type (we assume for simplicity that $\mu = \nu = \frac{1}{4}$). We then add the immigrant gamete probabilities to the gamete mass measurement, and renormalise so that female gamete frequencies sum to one, and so do male gamete frequencies. This gives us the initial gamete frequencies. As an example, in the case of a single infected male immigrant of genotype *AASS*, the initial gametic frequencies are:

Infected female *AS*: 0
Infected female *aS*: 0
Infected female *As*: 0
Infected female *as*: *J*
Uninfected female *AS*: 0
Uninfected female *aS*: 0
Uninfected female *As*: 0
Uninfected female *as*: 1 – *J*
Infected male *AS*: (2 - *J*)/(2 – *J* + (1 - *J*)*N*)
Infected male *aS*: 0
Infected male *As*: 0
Infected male *as*: 0
Uninfected male *AS*: 0
Uninfected male *aS*: 0
Uninfected male *As*: 0
Uninfected male *as*: (1 - *J*)*N*/(2 – *J* + (1 - *J*)*N*)

Local LD

Modelling local linkage disequilibrium is similar to above, with the added complexity that there are now two neutral loci to keep track of rather than just one. We label the alleles loci *A/a*, *B/b*, and *S/s* (the suppressor). Thus there are now 27 basic genotypes (3^3, since there are 3 loci, and three different possible genotypes at each one).

We now have to separately consider recombination between the suppressor and the two neutral loci (occurs at a rate r_1), and recombination the two neutral loci (occurs at a rate r_2). We make the simplifying assumption that these recombination events occur independently of one another, which is probably a reasonable assumption as long as the two rates aren't too similar in size.

The final complication is that with so many more genotypes to keep track of, there are many more types of heterozygotes. In line with our calculation of μ and ν above, we have to calculate the frequency of each type of gamete being passed on by a given heterozygote.